Two laboratory methods for diagnosis of herpes simplex keratitis

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SUMMARY Two techniques are described which enable a rapid diagnosis of herpes simplex keratitis to be made. The tests, antibody/antigen reactions, were shown to be accurate and sensitive in the 119 patients examined. A result is available within four hours with the indirect peroxidase-antiperoxidase method and within one hour with the direct method. The techniques are relatively inexpensive, though labour intensive. Negative reactions were found in treated cases and in those with some delay in the histochemical staining.

In many cases of herpes simplex keratitis the clinical findings are pathognomonic, and there is little doubt about the diagnosis. There are a number of situations, however, where one may suspect herpes simplex virus to be the causative factor when the picture is not typical.1 These patients present a diagnostic problem, and the danger is that they will be started on therapy which may be inappropriate and possibly toxic to an already damaged cornea. In addition, such treatment may so confuse the clinical picture that a correct diagnosis is impossible at a later date. Such cases require, therefore, a laboratory diagnosis.

Various techniques are used to establish a diagnosis of herpes simplex virus infection. Culture tests for virus isolation produce a variable yield,14 and the technique is difficult and time consuming, requiring two to five days. Serological examination provides, at best, confirmatory evidence, and in addition a high antibody titre is not normally achieved, for up to 90% of the population have a low titre owing to previous infection.9 Direct demonstration of the agent in exfoliated cells is generally less sensitive than cell culture; the smears must be of high quality and the virus inclusions are difficult to detect. The measurement of tear antibodies may be helpful but is time consuming.7 Electron microscopy of cells taken from infected tissue is accurate,4 but the procedure is expensive, slow, and of limited clinical value.

We describe two immunocytochemical tests which are rapid, simple, and reasonably inexpensive.

Patients and methods

One hundred and nineteen patients were examined to assess the accuracy, predictability, and usefulness of the tests.

The initial technique used was the indirect peroxidase-antiperoxidase (PAP) corneal smear method, with which we examined 58 patients over the age of 10. During a six-month period patients who presented with herpetic corneal lesions, both treated and untreated, and corneal ulcers of doubtful aetiology were investigated. On presentation to the External Eye Disease Clinic scrapings were made from the epithelial lesions by one of the three clinicians involved. Amethocaine topical anaesthesia was used, and under microscopic control the tissue was removed with a Bard-Parker blade. Great care was taken to ensure that an adequate amount of material was obtained and that it was spread evenly on the slides. Two smears were obtained from each patient, one being used as a control. The eye was padded for 24 hours following the procedure. The slides were transported to the laboratory for immediate testing.

In the second part of the study we examined 61 patients with the same inclusion criteria as the first group. Smears were taken in a similar manner, but the direct immunoperoxidase method was used instead.
LABORATORY METHODS
Smears were made on perfectly clean slides by a streaking technique to achieve separated and flattened cells. This area was outlined with a diamond pencil and smears were then fixed in acetone or Cytofix for five minutes. They should be stained as soon as possible after receipt in the laboratory.

PAP TECHNIQUE
The endogenous peroxidase was removed by treating smears in 3% H₂O₂ (100 vols) in methanol for 20 minutes. The slides were gently rinsed with trometamol (TRIS) saline, pH 7-6, and left standing in this for five minutes. The excess liquid was carefully wiped away from around the smear area. Four to 6 drops of normal swine serum diluted 1/10 in trometamol buffer, pH 7-6, were then applied and incubated for 20 minutes. The serum was then tapped off and the excess wiped away. Four to 6 drops of rabbit antibody to herpes simplex virus type 1, diluted 1/250 in trometamol buffer were applied, and incubated for 20 minutes. The slides were washed gently about six times in trometamol saline and left standing in the final rinse for five minutes. The excess liquid from around the smear was wiped away. Four to six drops of swine antibody to rabbit immunoglobulin diluted 1/100 were applied. The slides were gently washed well with trometamol saline, and left standing for five minutes in the final rinse before excess liquid was wiped away. Four to 6 drops of the PAP complex diluted 1/100 were added, and the slides were then incubated for 20 minutes before being washed well with trometamol saline.

All sera reactions were carried out in a humidity chamber at approximately 30°C. The substrate solutions used to give the coloured results for interpretation were as follows.

(1) Diaminobenzidine tetrahydrochloride (DAB) was used in 30 cases. This was prepared immediately before use by dissolving 6 mg of 3,3-diaminobenzidine tetrahydrochloride in 10 ml of 0-05 M trometamol buffer, pH 7-6, with the addition of 0-1 ml of 3% H₂O₂ (100 vols). The solution was filtered if precipitation occurred. The application time was five minutes at room temperature. After the application of DAB substrate 4 to 6 drops were placed on each smear. The slides were then rinsed with distilled water, counterstained with Mayer’s haematoxylin for three minutes, and blued in running water for five minutes, dehydrated, cleared, and mounted in synthetic resin.

(2) Aminoethylcarbazole (AEC) was used in 28 cases. 4 mg of 3-amino-9-ethylcarbazole was dissolved in 1 ml N,N dimethylformamide and added to 14 ml of 0-1 M acetate buffer, pH 5-2. 0-15 ml of 3% H₂O₂ (100 vols) was added, and this was then filtered on to the slides. The application time was 45 minutes at room temperature. Slides were then counterstained with Mayer’s haematoxylin and hydromounted.

The controls for the substrates were fresh normal blood films fixed in ethyl alcohol and 10% formalin. The positive control used was a tissue section infected with herpes simplex virus, and the negative control was a duplicate corneal smear substituting non-immune rabbit serum for rabbit antibody serum.

RAPID DIRECT METHOD FOR CORNEAL SMEARS
The smears were made and fixed as in the PAP technique. They were then treated with 3% H₂O₂ in methanol for five minutes. The slides were washed gently with distilled water and finally with trometamol saline and left standing in it for five minutes. Excess liquid was carefully wiped away from around the smear area. Four to 6 drops of a protein blocking agent (0-1% bovine albumin, 0-1% immunoglobulin, 0-1% gelatin in 0-05 M trometamol buffer, 15 mM sodium azide as preservative) were added and left for five minutes. Excess liquid was wiped away. Four to 6 drops of peroxidase conjugated rabbit immunoglobulin were added to human herpes simplex virus type 1 diluted 1/50 in trometamol buffer, pH 7-6, and left for 20 minutes. The slides were then washed well with distilled water and treated with the aminoethylcarbazole substrate for 30 minutes. They were then washed well with distilled water and stained with Mayer’s haematoxylin for three minutes, blued in running water for five minutes, and mounted in glycerol gel.

The control for the AEC substrate was a fresh normal blood film fixed in ethyl alcohol and 10% formalin. The negative control used was a duplicate corneal smear treated similarly as the test smear, substituting rabbit immunoglobulin in herpes simplex virus negative serum for the peroxidase conjugated sera.

Results
No clinical information was given to the pathologist and only at the end of the study was the clinicopathological correlation made. Fifty-eight smears

<table>
<thead>
<tr>
<th>Table 1 PAP method</th>
<th>Number</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Patients</td>
<td></td>
<td>All positive</td>
</tr>
<tr>
<td>Definite dendritic ulcers (untreated)</td>
<td>58</td>
<td>All positive</td>
</tr>
<tr>
<td>Definite dendritic ulcers (treated)</td>
<td>36</td>
<td>All negative</td>
</tr>
<tr>
<td>Controls (1 abrasion, 1 herpes zoster ophthalmicus, 5 bacterial ulcers, 6 with delay in processing)</td>
<td>9</td>
<td>All negative</td>
</tr>
</tbody>
</table>
were examined by the PAP method (Table 1) and 61 by the rapid direct method (Table 2).

The preparations examined by the DAB method gave the typical distinct yellow to brownish reaction in the cells containing antigen. Haematoxylin was the counterstain, and the negative cells stained in the conventional way with it. The staining, when the method is executed properly, was never equivocal. Occasional weak non-specific staining was present but this was easily interpreted with experience. AEC positively reacting cells containing herpes simplex virus type 1 antigen gave a clear orange tone in the cytoplasm (Fig. 1), and again negative cells stained only with haematoxylin. Non-specific staining was not found.

All patients with untreated dendritic keratitis had a positive result both by the PAP method and the direct corneal smear method.

A total of 27 patients were on antiviral therapy when corneal scrapings were done. The duration of therapy ranged from one to 14 days. The results were negative in all patients by both methods.

For technical reasons there was a delay in processing of the material taken from seven patients. The time interval was six days to 25 days. All of these specimens were unsatisfactory and autolysis was noted.

Twenty patients were clearly clinically non-herpetic. In all of these the test was negative, as expected.

Table 2  Direct method

<table>
<thead>
<tr>
<th>Number</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Patients</td>
<td>61</td>
</tr>
<tr>
<td>Definite dendritic ulcers (untreated)</td>
<td>29 All positive</td>
</tr>
<tr>
<td>Definite dendritic ulcers (treated)</td>
<td>18 All positive</td>
</tr>
<tr>
<td>Controls (6 abrasions, 7 bacterial ulcers, 1 delay in processing)</td>
<td>14 All negative</td>
</tr>
</tbody>
</table>

Discussion

In a preliminary report on a small number of patients we suggested that an antibody/antigen histochemical test might be useful in establishing an early diagnosis in herpes simplex virus keratitis. This larger series confirms our initial impression that it is an accurate, reliable and useful test (method 1). A similar method was reported in assessing a tear film aspirate in a patient with a dendritic ulcer. We have recently modified the technique whereby a result is obtained within one hour (method 2).

All corneal smears taken from patients with dendritic ulcers on antiviral therapy (duration one to 10 days) were negative by the indirect PAP and direct methods. This is of some interest, as it suggests that, while there is still some ulceration present, the antiviral agent had neutralised the virus antigen.

The 20 controls had negative results in all cases. This, coupled with the fact that all the untreated dendritic ulcers gave a positive result, suggests that the tests are reliable and can be used diagnostically in corneal ulceration.

In seven patients the unscheduled delay of more than three days in processing and examining the specimens produced interesting results. In all cases the material proved unsuitable and the results were negative. Slides can be stored at 2–4°C in a trometamol buffer bath, pH 7–6, for up to three days, and other methods of storage are being studied. It was found that no antibody/antigen staining took place after that time, and that the cells appeared morphologically to disintegrate. Either acetone or Cytofix was suitable for fixation and did not interfere with the reaction. Technical accuracy in performing these tests is essential for reproducibility.

From the clinical point of view the corneal scrapings must be carefully taken. There is no ethical problem in doing this, as one of the recognised methods of treatment of epithelial disease of the cornea is debridement. The material must be spread very thinly and evenly on the slide in order to get the best results. If the material is thickly spread, clumping of the cells occurs, and it is difficult for the pathologist to process and interpret the results. The technique should be strictly adhered to, and carried out precisely, in order to produce the best specimens for examination.

We suggest, therefore, that the tests described above provide a reliable, rapid, and inexpensive method of assessing corneal ulceration and can be used as one of the investigations to establish a positive diagnosis in corneal ulceration. Comparisons were not made with other laboratory techniques such as cell culture or immunofluorescence staining, as it was considered that the
controls (25 in number) sufficiently supported the hypothesis regarding the reliability and specificity of the techniques described.

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References


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